Histopathological Changes and Inflammatory Signaling Elevation in Distant Organs of Rats Following Experimental Periodontitis Induction

Fazle Khuda[1], Badiah Baharin[2], Nur Najmi Mohamad Anuar[3], Nurrul Shaqinah Nasruddin[1]*

1. Dept. of Craniofacial Diagnostics and Biosciences, Faculty of Dentistry, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia.
2. Dept. of Restorative Dentistry, Faculty of Dentistry, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia.
3. Faculty of Health Science, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia.

Abstract

Periodontitis is a chronic inflammatory condition that is linked to several systematic diseases. The precise roles and mechanistic aspects of the link between periodontitis and systemic diseases are still up for discussion because of the diversity of the responses and the variability of the evidence. This study aims to assess the inflammatory condition by analyzing the proinflammatory cytokines (IL-1β, IL-6, and TNF-α) expression and histologically examining periodontium, gingival, heart, and kidney tissues after experimental periodontitis induction.

Twelve rats were divided into 3 different groups: the control group (0-day), and the experimental groups (7-days and 14-days). A sterile 0.2 mm wire was placed into the interdental area of the right maxillary 1st and 2nd molar teeth, and 0.5µl of *Enterococcus faecalis* suspension containing $1.5x10^8$ CFU/ml of bacteria were administered into the gingival sulcus of rats in the 7- and 14-days groups. The rats were euthanized after the induction period, respectively. Samples from maxillary jaw, gingiva, heart, and kidney tissue were collected for RT-qPCR assay and histological analysis. The results show that IL-1β, IL-6, and TNF-α expression is upregulated in gingival, heart, and kidney tissue samples at 7 and 14 days when compared to the control group. Furthermore, inflammatory cell infiltration in the periodontium, heart and kidney tissue samples has also been observed.

The study suggests an inflammatory response within the periodontal tissue, heart, and kidney following experimental periodontitis induction. As a result, periodontitis may cause inflammation in distant organs like the heart and kidney.

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Introduction

Periodontitis is a chronic inflammatory condition that affects the periodontium and is brought on by the interaction of periodontal pathogenic bacteria and the host immune response, leading to the production of proinflammatory mediators and tissue destruction. [1] Periodontal disease is linked to a number of systematic diseases such as cardiovascular disease, diabetes, chronic kidney disease, stroke, rheumatoid arthritis, and oral cancer. [2,3] Cytokines are the peptide mediators that play an essential role in cell signaling and communication. Cytokine regulates cell proliferation, immunological responses, differentiation, and inflammatory responses. The major proinflammatory cytokines that are increased in response to the periodontal inflammatory reaction are IL-1β, IL-6, and TNF-α. [4] There are many disease-related effects of local inflammation disruption. [5] This could occur because of the inflammatory cascades linked to periodontitis, in which locally generated pro-inflammatory mediators enter the bloodstream and travel to other organs, disrupting the equilibrium of the inflammatory state. [6]

Understanding how systemic inflammation and oral inflammation in periodontal disease interact can help explain the long-term negative effects that inflammation may have on the function of various organs. This might aid in understanding how much oral disease increases the chance of getting non-oral disorders. Although several studies have reported on the effect of periodontitis on cardiovascular disease,

Corresponding author:
Dr. Nurrul Shaqinah Nasruddin,
PhD. Department of Craniofacial Diagnostics and Biosciences, Faculty of Dentistry, Universiti Kebangsaan Malaysia,
Jalan Raja Muda Abdul Aziz, 50300, Kuala Lumpur, Malaysia.
E-mail: shaqinah@ukm.edu.my

there is little information on the impact of periodontitis on kidney disease. To better understand the inflammatory response in kidney, heart, and gingival tissue samples, this study will evaluate the expression of proinflammatory cytokines (IL-1, IL-6, and TNF-α) and histological changes after experimentally inducing periodontitis.

Materials and methods

Animal Care

The experiment was carried out in accordance with University Kebangsaan Malaysia Animal Ethical Committee's (UKMAEC) approval (FD/2018/NURRUL SHAQINAH/28-NOV./967-NOV.-2018-JAN. -2020). All experiments were conducted according to UKMAEC regulations that at least complied with the requirements of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Washington, DC: National Academy Press, 1996). The ARRIVE 2.0 reporting standards are followed for reporting studies. [7] Sample sizes were calculated depending on an ANOVA calculation using the degree of freedom that has an "E" value that must be between 10 and 20. [8] We obtained 12 Sprague Dawley (SD) male rats 6 weeks old and weighing on average ~180 g raised under pathogen free environments in the Laboratory Animal Resource Unit, University Kebangsaan Malaysia. Rats were kept in the similar conditions (25 ± 1 °C, 55% humidity, 12/12, light/dark cycle) and given adequate water and food. The rats were divided into three different groups, with four rats (n=4) per group: control (0-day), experimental (7-days), and experimental (14-days).

Induction of Experimental Periodontitis

Experimental periodontal disease induced by the insertion of a "(" shaped sterile orthodontic 0.2 mm wire of 5 mm length with the help of a needle holder between the interdental area of the right maxillary 1st and 2nd molar teeth. [9] General anesthesia was given with a combination of 10% ketamine at 100 mg/kg and 2% xylazine at 10 mg/kg body weight intraperitoneally. The McFarland standard was used to standardize 1.5 x 10^8 CFU/ml of bacteria from the *E. Faecalis* strain (ATCC 29212, USA). A Hamilton syringe was used to inject 0.5 µl of bacterial suspension into the gingival sulcus of the right maxillary first and second molar teeth areas following the placement of a ligature wire. Rats were observed for at least an hour daily for behavioral and physical signs of animal welfare, and the rats met the standards for an acceptable level of humane treatment with no signs of pain or discomfort other than those caused by the experimental stimulus. At the end of the experiments, all rats were humanely euthanized at their respective induction periods by anesthesia overdose (10% ketamine at 200 mg/kg and 2% xylazine at 20 mg/kg) followed by cervical dislocation.

Histological Processing & Descriptive Analysis

Samples from the maxilla, heart, and kidney tissues were obtained, fixed, processed, and stained with hematoxylin and eosin (H&E) accordingly for histological assessment. The soft tissues for histopathological examination were immediately fixed with 10% neutral buffered formalin for at least 48 hours. The maxillary specimens were decalcified in a 10% buffered ethylenediaminetetraacetic acid (EDTA, Sigma) solution for three weeks. A rotary microtome (Leica RM2135, Germany) was used to cut samples into 5 µm thickness. Maxillary specimens were cut coronally perpendicular to the long axis of the alveolar ridge. Each included a complete cross section of the teeth, bone, and soft tissue.

Quantitative Real-Time PCR Assay

Gingival tissue around the molar area, heart, and kidney tissue samples were collected for RNA isolation. The Innu PREP® RNA Mini Kit 2.0 (Analytikjena) was used to extract and purify 20 mg of RNA from the gingival, heart, and kidney tissue as instructed by the manufacturer. The concentration and purity of the isolated total RNA were then evaluated using a spectrophotometer, NanoDrop (ND-2000, Thermo Scientific). The study excluded RNA samples with an absorbance 260/280 ratio lower than 1.8. Following integrity confirmation, the RNA was transcribed to cDNA by the ReverTra Ace® qPCR RT with gDNA Remover (TOYOBO) kit per the manufacturer's instructions. The IL-1β, IL-6 and TNF-α genes of 10 µl reaction were amplified quantitatively using Real-Time PCR (RT-qPCR) using the ChamQ Universal SYBER® qPCR Master Mix (Vanzyme). As shown in Table 1, the forward and reverse primers were used. [10] The targeted gene sequence was amplified using a Real-Time PCR thermal cycler (Bio-Rad CFX96 Connect™, USA).

Pre-denaturation at 95°C for 30 sec, denaturation at 95°C for 10 sec, and annealing at 60°C for 30 sec, for 40 cycles were the two step amplification methods that were employed. Every RT-qPCR assay included a no-template control to check for contamination. All reactions were performed in triplicate. The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a housekeeping gene. The Livak method $2^{-\Delta\Delta CT}$ was used to analyze the data fold expression. [11]

Statistical Analysis

IBM SPSS Data Editor version 23.0 (IBM, USA) was used to perform the analysis of raw data, and the values were shown as the mean (±) and standard errors or standard deviation. A one-way ANOVA analysis was combined with Tukey's post hoc test to assess the differences between different groups mean, and a significance level of less than 0.05 was determined.

Gene name	Forward (5'-3' primer sequence)	Reverse (5'-3' primer sequence)
IL-1β	TTCATCTCGAAGCCTGCAGTG	GACCTGTTCTTTGAGGCTGAC
IL-6	TAGCCACTCCTTCTGTGACTCTAACT	GACTGATGTTGTTGACAGCCACTGC
TNF-α	CCCATGTTGTAGCAAACCCTC	TATCTCTCAGCTCCACGCCA
GAPDH	TGCTGGTGCTGAGTATGTCG	ATTGAGAGCAATGCCAGCC

Table 1: Primer sequence for RT-qPCR assay.

Results

Expression of IL-1β, IL-6 and TNF-α in Gingival Tissue, Heart, and Kidney Tissue Samples

RT-qPCR was carried out to investigate the IL-1β, IL-6 and TNF-α mRNA expression. The findings are presented in Figure 1. In gingival, heart, and kidney tissue samples, the current investigation shows that all three proinflammatory cytokines mRNA expression was upregulated following their respective induction periods as compared to the control group. The mRNA expression of IL-1β, IL-6 and TNF-α upregulated significantly ($p < 0.05$) in gingival tissue samples at 7-days and 14-days which suggest inflammatory response of periodontal tissue. However, although not statistically significant ($p > 0.05$), IL-1β, IL-6, and TNF-α mRNA expression was upregulated in heart tissue samples during both induction periods. Furthermore, significant upregulation of IL-1β ($p < 0.01$), IL-6 ($p < 0.01$) and TNF-α ($p < 0.05$) at 7-days post induction and IL-1β ($p < 0.05$), IL-6 ($p < 0.01$) at 14-days post induction in kidney tissue samples have been observed in the study.

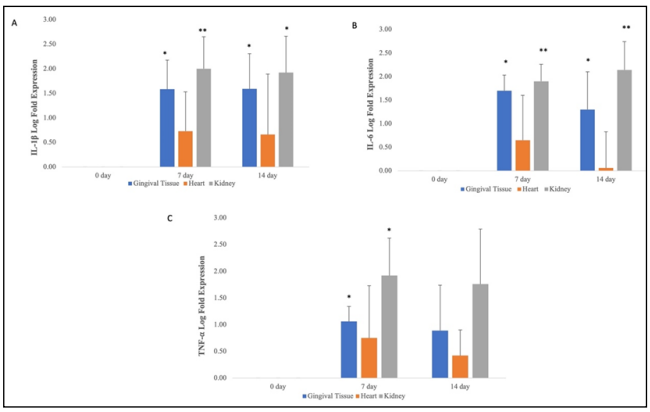

Figure 1. IL-1β, IL-6 and TNF-α mRNA expression level in the gingival, heart and kidney tissue samples shows upregulation of three proinflammatory cytokines at both induction period. Data are shown as the mean ± standard deviation.

Descriptive Histological Analysis

The histopathological findings were described qualitatively, and the descriptions were compared between the groups and shown as Figure 2.

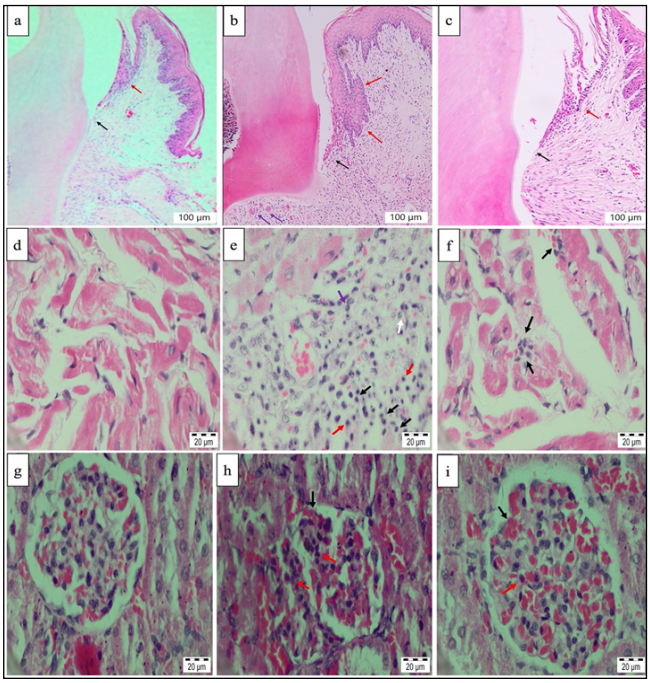

Figure 2. Histological analysis shows in periodontium hyperplastic junctional epithelium migrated towards the apical region (red arrow), attachment loss from cementoenamel junction (black arrow), infiltration of the inflammatory

cells, multinucleated giant cells representing the osteoclast (purple arrow) [b], In the heart, infiltration of the mixed inflammatory cells is composed of mostly neutrophils (black arrow), eosinophils (purple arrow), a few multinucleated giant cells (white arrow), and mononuclear inflammatory cells (red arrow) [e], In the kidney, inflammatory cells infiltration (red arrow), congested blood vessels within the glomeruli, and compression of the Bowman space (black arrow) [h], with 40x magnifications. The control group samples (a, d, g) showed no histopathological changes.

The periodontium of the control group shows normal junctional epithelium with no apical migration and attachment loss from the cementoenamel junction (CEJ) and no inflammatory cells infiltration (a). However, at 7-days post induction (PI) group showed hyperplastic junctional epithelium migrating towards the apical region, attachment loss from the cementoenamel junction (CEJ), infiltration of the inflammatory cells, and multinucleated giant cells representing the osteoclasts (b). In the 14-days PI group, inflammatory cells infiltration was less as compared to the 7-days PI group, even though thin ulcerated junctional epithelium and attachment loss were observed (c). In heart tissue samples, no histopathological changes within the myocardium were observed in the control group 0-day (d). In the 7-days PI group, however, infiltration of mixed inflammatory cells mostly neutrophils, eosinophils, a few multinucleated giant cells, and mononuclear inflammatory cells with necrotic myocardium was observed (e). In the 14-days PI group, less inflammatory cells were observed as compared to the 7-days group (f). No histopathological changes within the kidney were observed in the control group 0-day (g). However, inflammatory cells infiltration within the interstitial space and glomerulus, congested blood vessels within the glomeruli, and compression of the Bowman space were observed in the 7-days PI group (h). In 14-days PI group, congested blood vessels and few inflammatory cells were present (i).

Discussion

In the current study, experimental periodontal disease induced by concurrent infection with ligature wire and *E. faecalis* inoculation to assess the expression of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) and histopathological changes in the periodontium, heart, and kidney tissue samples. Because the heart is a crucial organ that receives blood from all over the body and the kidney is a distant organ that eliminates toxins from the body, we have chosen to study the inflammatory response in these two distant organs after experimental periodontal disease induction. Multiple types of microorganisms can cause periodontal disease, thus it's important to determine how the condition affects those key organs. Interestingly, in both experimental groups, we found upregulation in the IL-1β, IL-6 and TNF-α expression and histopathological changes when compared to the control group.

In the inflammatory phase of periodontal disease, cytokines are vital. Proinflammatory cytokines drive the immune response against the invading microorganisms by maturing dendritic cells, increasing the phagocytes' ability to kill bacteria, and bringing new innate cell populations into the infection sites. After successful colonization of the gingival surface, the pathogens proliferate in the host by the PAMP/PRR pathway and induce immunological responses in circulating immune cells and local resident cells that result in the production of proinflammatory cytokines. The elevated expression of proinflammatory cytokines is an important hallmark of inflammatory events in heart and kidney conditions. In the present study, the expression of these proinflammatory cytokines indicated the initiation of the inflammatory process in the gingiva, heart, and kidney due to experimental periodontitis induction. Due to microbial challenge, inflamed gingival tissue are characterized by increased number of IL-1β secreting cells such as neutrophils and macrophages. [12] After secretion in response to pathogenic stimulus, IL-1β responsible for increased blood flow, leucocyte recruitment and neutrophil infiltration. [13] Following tissue injury by causative stimuli, activation of the inflammasome causes a local surge of IL-1β, which dramatically enhances the inflammatory response and recruits more inflammatory cells. IL-6 locally produced in inflamed tissues from neutrophil, macrophages, T cell, fibroblast in response to bacterial challenge or other cytokines like IL-1β or TNF-α. [14] TNF-α stimulates macrophage and neutrophil

chemotaxis, cytotoxic activity, phagocytic and facilitates leukocytosis by increasing the expression of intracellular adhesion molecules and endothelial leukocyte adhesion molecules at inflammatory locations.

In this study, ligature wire acts as a plaque-retentive factor, allowing periodontal pathogen to colonies at the induction site. *E. Faecalis,* together with the periodontal pathogens contributed to induce periodontitis. A histological examination of the periodontium in the 7-days group reveals the presence of innate immune cells that produce pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α). The Rt-qPCR assay of the gingival tissue reveals a significant upregulation in the expression of three proinflammatory cytokines. Furthermore, the presence of inflammatory cells in heart and kidney indicates an innate immune response in both organs that gives rise to upregulation in the expression of three proinflammatory cytokines that initiated the inflammatory events too. Nevertheless, despite the fact that the same stimulus was given for both induction periods, all inflammatory responses were more evident in the 7-days group. At the end of the 14-days induction period, inflammatory symptoms reduced, most likely as a result of pathogen clearance by innate immune cells, which was more noticeable on the 7-days. De Molon et al. (2014) have reported a consequent reduction in the inflammatory condition with time following experimental periodontal disease induction. [15] One of the study's limitations is that although systemic proinflammatory cytokine levels can be influenced by other conditions, no biochemical analysis was done to measure them; instead, the study concentrated on the local inflammatory response in these three organs. However, this research suggests that periodontal disease can result in an inflammatory condition in a distant organ, providing important information for treating patients with heart and kidney diseases who also have periodontal disease.

Conclusions

The findings of the current study indicate that the ongoing microbial challenge in the periodontal tissue may result in inflammation of the heart and kidney. Due to the condition, other organ systems may become overburdened. More research is needed to better understand the relationship.

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Declaration of Interest

The authors report no conflict of interest.

References

1. Khuda F, Anuar, NNM, Baharin, B, Nasruddin, NS. A mini review on the associations of matrix metalloproteinases (MMPs) -1, -8, -13 with periodontal disease. AIMS Mol Sci. 2021;8(1): 13–31.
2. Hajishengallis G, Chavakis T. Local and systemic mechanisms linking periodontal disease and inflammatory comorbidities. Nat Rev Immunol. 2021;21(7): 426–440.
3. Susanto A, Carolina DN, Sopiatin, S, Hendiani I. The association between periodontitis and atherosclerotic cardiovascular disease: A scoping review. J Int Dent Medical Res. 2022;15(2): 909-916.
4. Pan W, Wang Q, Chen Q. The cytokine network involved in the host immune response to periodontitis. Int J Oral Sci. 2019; 11(3): 30.
5. Valentine J, Saladyanant T, Ramsey K, Blake J, Morelli T, Southerland J, et al. Impact of periodontal intervention on local inflammation, periodontitis, and HIV outcomes. Oral Dis. 2016;22, 87–97.
6. Hajishengallis G. Periodontitis: from microbial immune subversion to systemic systemic inflammation. Nat Rev Immunol. 2015;15, 30–44.
7. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, et al. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. PLoS Biol. 2020;18: e3000410.
8. Ilyas MN. Sample size calculation for animal studies using degree of freedom (e); an easy and statistically defined approach for metabolomics and genetic research. Curr Trends in Biomed Eng Biosci. 2017;10(2): 2–3.
9. Li D, Feng Y, Tang H, Huang L, Tong Z, Hu C, et al. A simplified and effective method for generation of experimental murine periodontitis model. Front Bioeng Biotechnol. 2020;8(May): 1–10.
10. Kuo PJ, Fu E, Lin CY, Ku CT, Chiang CY, Fu MM, et al. Ameliorative effect of hesperidin on ligation-induced periodontitis in rats. J Periodontol. 2019;90(3): 271–280.
11. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 2001;25(4): 402–408.
12. Trombelli L, Scapoli C, Carrieri A, Giovannini G, Calura G, Farina R. (2010). Interleukin-1β levels in gingival crevicular fluid and serum under naturally occurring and experimentally induced gingivitis. J Clin Periodontol. 2010;37(8): 697–704.
13. Cheng R, Wu Z, Li M, Shao M, Hu T. Interleukin-1β is a potential therapeutic target for periodontitis: A narrative review. Int J Oral Sci. 2020;12(1): 2.
14. Tawfig, N. 2016. Proinflammatory cytokines and periodontal disease. Journal of Dental Problems and Solutions. 2016;7: 12–17.
15. De Molon RS, de Avila ED, Boas Nogueira AV, Chaves de Souza JA, Avila-Campos MJ, de Andrade CR, et al. Evaluation of the host response in various models of induced periodontal disease in mice. J Periodontol. 2014;85(3): 465–477.